Hydroalcoholic extract of Centella asiatica and madecassic acid reverse depressive-like behaviors, inflammation and oxidative stress in adult rats submitted to stress in early life

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Abstract

Major depressive disorder (MDD) is a severe disorder that causes enormous loss of quality of life, and among the factors underlying MDD is stress in maternal deprivation (MD). In addition, classic pharmacotherapy has presented severe adverse effects. *Centella asiatica (C. asiatica)* demonstrates potential neuroprotective but has not yet been evaluated in MD models. Objective: This study aimed to evaluate the effect of *C. asiatica*extract and the active compound madecassic acid on possible depressive-like behavior, inflammation, and oxidative stress in the hippocampus and serum of young rats submitted to MD in the first days of life. Method: Rats (after the first day of birth) were separated from the mother for three hours a day for ten days. These animals, when adults, were divided into groups and submitted to treatment for 14 days. After the animals were submitted to protocols of locomotor activity in the open field and behavioral despair in the forced swimming test, they were then euthanized. The hippocampus and serum were collected and analyzed for the inflammatory cytokines and oxidative markers. Results: The *C. asiatica* extract and active compound reversed or reduced depressive-like behaviors, inflammation in the hippocampus, and oxidative stress in serum and hippocampus. Conclusion: These results suggest that C. asiatica and madecassic acid have potential antidepressant action, at least partially, through an anti-inflammatory and antioxidant profile.

1 Introduction

Major Depressive Disorder (MDD) is a severe disorder that causes enormous damage to people's quality of life and is one of the most prevalent forms of mental illness [1]. Studies have observed that childhood stress is one of the most potent phenomena in precipitating the expression of a genotype predisposing to MDD [2, 3]. MDD has a multifactorial etiology, which may include traumatic events and chronic stress in early and adult life, and may be accompanied by several comorbidities, such as metabolic and cardiovascular diseases, and chemical dependence, among other factors that can drastically reduce the quality of life of the people affected. Patients suffering from severe depression have high levels of morbidity and mortality, with profound economic and social consequences [4, 5].

Statistics from the World Health Organization (WHO) show that Major Depressive Disorder (MDD) affected more than 300 million people worldwide in 2017 and contributed to the highest percentage of disabilities. MDD is the leading cause of suicide deaths, contributing to 800,000 suicides annually. Data show that in 2015 suicide was the second leading cause of death among 15-29-year-olds worldwide [6].

Alteration in the functioning of neurotransmission systems is an essential characteristic of MDD, and classic antidepressant treatments have neurotransmitter control as the primary mechanism. The pathophysiology of MDD involves decreased brain levels of serotonin, norepinephrine, and dopamine, and this situation contributes to the behavioral symptoms characteristic of the disorder [7, 8]. One of the brain regions vulnerable to stress and MDD is the hippocampus, which is related to the modulation of emotions and regulation of the hypothalamic-pituitary-adrenal (HPA) axis. In MDD, the hippocampus has high inflammatory levels, reduced neuronal plasticity, and reduced hippocampal volume [9–11].

Among several biological phenomena, many studies have highlighted that changes in the oxidative balance are involved in the pathogenesis of MDD [12–16]. In addition, maternal deprivation (MD) stress can cause dysregulation in oxidative balance parameters, leading to oxidative stress in brain regions involved with depression [17]. It is also important to emphasize that oxidative stress is related to the severity of MDD and treatment-resistant depression (TRD) [18]. Among the various mechanisms in which oxidative stress can exert influence are modifications in various biological molecules, activation of different transcription factors, and consequent increase in anti- and pro-inflammatory cytokines [19]. On the other hand, through the activation of inflammatory cells, patients with MDD have increased oxidative stress markers [20] and pro-inflammatory cytokines [21], such as interleukins (IL) (IL-1, IL-2, and IL-6) and tumor necrosis factor-α (TNF-α) [22].

A fundamental aspect is that early life stress seems to be involved in the disorder's severity and the poor response to antidepressant treatments, both in humans [23] and in animals undergoing maternal separation protocols [24]. MD in animal models mimics chronic stress early in life, such as in situations of abandonment, abuse, and neglect [25]. MD induces depressive-like behaviors and biological changes that contribute to the pathophysiology of the disorder, such as neuroinflammation [26], and oxidative stress [27].

The portion of patients who adhere to treatment may resist the action of drugs, thus developing a depression resistant to the classic antidepressant treatment available in the clinic, or has several side effects. On the other hand, studies indicate that about 30–40% of patients end up not adhering to treatment [28, 29]. Besides stress in early life increases the risk of individuals developing MDD in adulthood, the individuals who develop depression in adulthood following chronic stress in early life are at greater risk of developing TRD [30]. Thus, it is clear the need to discover new strategies that make it possible to increase drug adherence and effectiveness [31]. In this context, pharmacological studies have intensified in recent years, focusing on substances extracted from plants, as well as synthetic derivatives of these natural compounds [32].

In this sense, it was possible to verify the importance of medicinal herbs as a drug option or auxiliary therapy in the treatment of MDD since it can cover many patients who have not been successful in classical approaches and considering that several plants have low toxicity and few side effects compared to drugs available in the clinic nowadays [33]. Among them, the species *Centella asiatica* (*C. asiatica*), popularly used for thousands of years, presents itself as an effective therapeutic strategy. Some studies have highlighted this medicinal species as a possible intervention and beneficial effect on MDD and neuronal plasticity [34]. Neuroprotective effects encompass several molecular and structural mechanisms, such as beneficial actions on the HPA axis and inflammation [35]. Studies on the extracts and active compounds of *C. asiatica* suggest its relevance as a therapeutic pharmacological strategy for MDD and its role in underlying biological mechanisms [36, 37]. Also, researchers observed that *C. asiatica* demonstrates anxiolytic and antidepressant effects [38], and anti-inflammatory effect by inhibiting the serum expression of tumor necrosis factor-α (TNF-α), interleukins (IL), IL-1β, IL-6, and immunoglobulin E (IgE) [39]. In rats undergoing olfactory bulbectomy, *C. asiatica* extract reversed

procedure-related depressive symptoms similar to the antidepressants imipramine, fluoxetine, and desipramine. In addition to reducing depressive-like symptoms, *C. asiatica* reduced anxious-like behavior in the elevated plus maze test [40].

In this context, this study was designed to be the first investigation evaluating the effect of hydroalcoholic extract from *C. asiatica* and the bioactive compound madecassic acid as having the potential to reverse or reduce depressive-like behaviors. In addition, we contributed to the pharmacological mechanism of the plant by observing the activity of the agents tested in the modulation of inflammatory markers and oxidative stress in the hippocampus and serum of rats.

2 Material and Methods

2.1 Chemical reagents and equipment

All chemical reagents used in this study were at analytical grade. Madecassic acid, purity > 95%, solid crystalline, was purchased from Cayman Chemical, Michigan, USA. Escitalopram oxalate, powder with purity > 95%, was purchased from Laborsan (Company Lepuge, São Paulo, Brazil). For spectrometric analysis a Multimode Plate Reader 96 microplate - SpectraMax® i3 was used (Molecular Devices, Sunnyvale, CA, USA).

2.2 Experimental design

This experimental research was approved by the Animal Ethics Committee (AEC), UNOCHAPECÓ, SC, under protocol code 002/CEUA/2021, and developed in a laboratory in partnership between the Federal University of Fronteira Sul (UFFS) and the Community University of the Chapecó Region (UNOCHAPECÓ). All the behavior tests were conducted according to the previous established protocols [41].

Figure 1 expresses an experimental scheme involving the MD protocol, pharmacological treatments, and behavioral tests. The animals were submitted to the MD protocol in the first ten days of life. When they reached 60 days, the animals were submitted to the chronic treatment of *C. asiatica* extract and madecassic acid for 14 days. The administration was performed by the gavage method. The 60 male Wistar rats were divided into 6 (six) groups (n = 10 for each group): Control without stress + vehicle (Control without stress); MD + vehicle (Stress + Control treatment); MD + Escitalopram (Stress + Positive control treatment) 10 mg/kg; MD + *C. asiatica* extract 30 mg/kg; MD + madecassic acid 10 mg/kg. The positive control escitalopram is a classic antidepressant of the selective serotonin reuptake inhibitor class [42], and the dose of 10 mg/kg is widely used in studies with previously published animal models [43–45].

A study in rats identified that doses of 10 and 30 mg/kg of C. asiatica per day chronically intranasally reversed the migraine caused by nitroglycerin and positively affected serotonin concentration [46]. Another study in rats analyzed a dose-response curve of 10, 30, and 100 mg/kg of C. asiatica extract. The results particularly highlighted doses of 10 and 30 mg/kg for achieving optimal memory

enhancement and related molecular changes increased hippocampal synaptic plasticity, with even more potent effects at a dose of 30 mg/kg [47]. Furthermore, the dose of 30 mg/kg orally is used in cosmetics in humans [48]. Therefore, the research's chosen dose of C. asiatica was 30 mg/kg.

Administration of 10 mg/kg of madecassic acid resulted in positive effects on the immune response of Labeo rohita fish against Argulus siamensis infection, modulating both the innate and adaptive immune responses, in addition to influencing the expression of genes related to the immune system [49]. The administration of 10 mg/kg of madecassic acid resulted in higher escape latency than scopolamine, indicating an effect on memory and learning in rats [50].

2.3 Plant material

C. asiatica were collected in Chapecó (SC), Brazil (27 ° 01 '55.14 'S and 52 ° 47 '29.42' W) in October 2021. The identity of the plant was verified by Professor Adriano Dias de Oliveira, curator of the Herbarium of the Community University of the Region of Chapecó (Unochapecó), where in a voucher specimen has been deposited (#4930).

2.4 Production of hydroalcoholic extract of *Centella asiatica* (HECa)

The leaves of *C. asiatica* were dried at room temperature (25 ± 5°C), ground in a knife mill (Ciemlab®, CE430), selected in a sieve (425 µm; 35 Tyler/Mesch), identified and stored with light protection. The extracts were produced by maceration (5 days) at room temperature, using dry milled leaves of the plant (100 g) and 70% ethanol (1:20, w/v). After filtration through a Büchner funnel, the hydroalcoholic extract of *Centella asiatica* (HECa) was concentrated via evaporation under reduced pressure, lyophilized, and stored at -20°C.

2.5 Chemical analysis of HECa

2.5.1 Mass spectrometry analysis (ESI-IT-MS^n)

A 10 ppm sample of HECa in MeOH Grade LC-MS was subjected to direct flow infusion performed on the Thermo LTQ XL (Thermo, San Jose, CA, USA), a Linear Ion trap mass spectrometer equipped with an electrospray ionization source (ESI), in positive and negative mode, under the following conditions; drying gas flow rate 8,0 L/min, capillary temperature 275 ºC, source voltage 4.0 kV, capillary voltage − 45 V, tube lens − 125 V and flow sample 10 µL/h. The fragmentations in the multiple stays (MS/MS) were performed using the collision-induced (CID) method at 28 eV.

2.5 *In vivo* experimental procedures

2.5.1 Maternal deprivation (MD)

The pups were deprived of the mother for 3 h/day in the first 10 days after birth. The MD consisted of removing the puppies from their mother's cage and keeping the litter together in another cage without

the mother. Non-deprived animals (controls) remained undisturbed in the original cage with their mother. The animals were weaned only on the 21st day after birth when they stayed under standard conditions. They were kept in 5 animals per cage, with a 12 hour light/dark cycle (from 7:00 am to 7:00 pm, with light from 7:00 am), with food and water *ad libitum*. The environment was maintained at a temperature of 23 ± 1°C.

2.5.2 Behavioral tests

All behavioral tests were performed in the morning (8:00–12:00 am), started 60 minutes after each treatment, and under a blinded observator to the experimental groups. The open field test assesses exploratory motor activity [51] (n = 10/group). The animals' locomotor activity was evaluated in the open field inside a box measuring 40 x 60 cm, surrounded by three wooden walls, a front glass wall, and a floor divided into 9 equal rectangles by black lines. The animals were allowed to explore the environment for 5 min. During that time, the crossings between the black lines were counted, and the number of times the rat was supported on its hind legs to explore the environment (rearings).

The forced swimming test assesses depressive-like behavior, as previously described by Porsolt et al. [52](n = 10/group). Each rat was placed individually in a cylinder with water at a temperature of 23ºC filled with enough water so that the animal could not rest its paws on the bottom. This test is performed over two days. On the first day (13th day of pharmacological treatment), the rats were forced to swim for 15 min (pre-test). On the second day of the test (14th day of pharmacological therapy), the rats were forced to swim for 5 min. Immobility parameters were evaluated, involving total immobility or movements to keep the head out of the water with no intention of escaping. Mobility parameters were also assessed, such as the time the animal spent swimming and the time it spent climbing the walls of the cylinder in an attempt to escape the environment.

2.6 Laboratory biochemical analysis

2.6.1 Total blood and tissue collection

After the last behavioral test (forced swimming), the animals were euthanized by decapitation. Immediately, 15 ml of whole blood was collected into a tube with separator gel. Then, the tube was centrifuged at 3500 rpm for 15 min to obtain serum samples. In sequence, a brain extraction was performed and the hippocampus was separated based on the histological description of Paxinos and Watson [53]. The hippocampus of each animal was placed in an individual microtubule and stored in an ultra-freezer at -80º C for further analysis.

2.6.2 Assessment of inflammatory cytokines

The levels of inflammatory cytokines IL-1β and IL-6 were assessed by the enzyme linked immunosorbent assay (ELISA) kits (Sigma-Aldrich, Darmstadt). The principle of these ELISA is based on an antibody sandwich format immune-colorimetric assay whose absorbance can be measured. Both IL-1β and IL-6 levels were analyzed using the kits manufacturers' protocols to serum and hippocampus. Given the solid

tissue mass, for the hippocampus firstly was necessary to perform digestion with 1.000 µl of TRIS HCl (50 mM) for 2 h and 30 min at room temperature, to obtain a supernatant and be used in the analyses. Protein quantification was performed using the Peterson's method (modified by Lowry) [54]. For the ELISA run, 100 µl of samples were added into microplate 96-well, covered, incubated overnight at 4°C, and then exposed to detection antibodies (100 µl) for 1 h at room temperature. After the plate washing, 100 µl of Streptavidin solution was added and incubated for 45 min at room temperature. Then, 100 µl of TMB one-step substrate reagent was added into wells, covered, and incubated for 30 min at room temperature in the dark. Finally, the reaction was stopped with 50 µl of stop solution and the reading was taken immediately at 450 nm of length-wave. The results were calculated considering the interpolation of the equation of the absorbance curve by the concentration and are expressed in picograms per milligram of protein (pg/mg).

2.6.3 Oxidative parameters

The oxidative stress markers were assessed on both serum and hippocampal samples. Serum samples were obtained from whole blood collected in an EDTA tube after centrifugation. For the hippocampal tissues, firstly samples were digested as described in the ELISA preparation to acquire a more fluid system. All the analysis was made at least in triplicates.

2.6.4 Myeloperoxidase (MPO) activity

MPO is a heme enzyme produced by inflammatory mediators and released from leukocytes at the site of injury; therefore, MPO reflects the activation of both neutrophils and lymphocytes. MPO catalyzes the reaction of chloride ions with H_2O_2 to generate large amounts of hypochlorous acid (HOCl), a reactive oxygen species that further reacts to generate singlet oxygen and hydroxyl radical. In the presence of H_2O_2 as an oxidizing agent, MPO catalyzes the oxidative coupling of phenol and 4-aminoantipyrine (AAP), originating a colored product, quinoneimine, with a maximum absorbance of 492 nm [55]. The MPO activity was analyzed using a modified peroxidase system, with mixing of 12 µl of sample with 148 µl of AAP in phenol solution (AAP 2.5 mM; phenol 20mM), and 17 µl of H_2O_2 solution (17 mM). After 30 min of incubation at 37 ºC, the system was read spectrophotometrically. The results were expressed as µM of quinoneimine per mg of protein produced in 30 min (µMq/mg/30 min).

2.6.5 Lipid peroxidation

Lipoperoxidations are extremely rapid reactions formed by the breakdown of polyunsaturated fatty acids, which are usually measured by their products, mainly thiobarbituric acid reactive substances (TBARS), among which malondialdehyde (MDA) is the main one [56]. To evaluate this product, the reaction of thiobarbituric acid (TBA) with samples was used, which in the presence of TBARS, results in a pink product that can be read at 532 nm. Briefly, 20 µl of samples were mixed with 55 µl of distilled water, 100 µl of orthophosphoric acid (0.2 M) and 25 µl of TBA (0.1 M). After 45 min of incubation at 37ºC, a spectrophotometric reading was taken. Results were expressed in nM TBARS/ml.

2.6.6 Determination of total thiol (PSH) and non-protein thiol (NPSH) levels

The protocol established by Ellman [57] with adaptations to determine both levels of total thiols and non-protein thiols. This method consists of the reduction of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and measured at 412 nm. For total thiol assay, 40 µl of sample was added in a 96-well plate and mixed with 200 µl of potassium phosphate buffer (PPB) (1 M, pH 6.8). Then, 20 µl of DTNB was added following the immediate reading. For non-protein thiols was carried out the same experimental procedure, except the samples were deproteinized with added equal sample volume of trichloroacetic acid (TCA) at 10% before analysis, and the 30 µl of remaining supernatant was used. The results were determined using a cysteine standard curve and expressed as µmol/l.

2.7 Statistical analysis

Statistical analysis was performed using GraphPad Prism 9 software. The Shapiro-Wilk test was employed to verify the data normality distribution. The differences between the groups in relation to the studied variables were evaluated through the variance analysis one-way ANOVA followed by Tukey's *post hoc* test. The differences in the probability of rejection of the null hypothesis at < 5% ($p < 0,05$) were considered statistically significant. All data are expressed as mean ± standard error, and statistical significance was defined for *p*-values of $*p < 0,05$, $**p < 0,01$, $***p < 0,001$, and $****p < 0,0001$.

3 Results

3.1 Chemical analyzes

3.1.1 Mass spectrometry analysis (ESI-IT-MS/MS)

HECa was analyzed by tandem mass spectrometry using an electrospray ionization source coupled to an ion trap mass spectrometer based on the direct infusion technique. Structures of the constituent compounds were determined based on their MS2 and MS3 fragmentation patterns and compared with literature data. Due to the parameters established in the test, it is not possible to detect madecassic acid (Fig. 2). However, analysis in the negative and positive modes of the concentrate revealed the presence of nine compounds, including phytoconstituents of the antioxidant class such as catechin and verbascoside (Table 1 and Fig. 3).

Table 1
Phytochemical analysis of hydroalcoholic extract from *Centella asiatica* (HECa) thought of spectrometric assays (ESI-IT-MS/MS).

Compound	$[M-H]^-$	MS_2	Reference
Catechin	289	187, 171, 161, 125	[104]
Ellagic acid	301	257, 272, 283	[105]
Rhamnetin	315	300, 271, 165, 121	[106]
Quercetin-dimethyl ether	329	314, 299, 285, 241	[107]
Kaempferol glucoside	447	285, 241, 257, 267	[106]
Chicoric acid	473	311, 293, 179	[105]
Caffeic acid rutinoside	487	469, 459, 441, 427, 179	[106]
Caffeoyl diglucoside	503	341, 179, 161, 143	[108]
Verbascoside	623	461, 315, 179	[109]

3.1 Effects of *C. asiatica* extract, madecassic acid and escitalopram treatment

3.1.1 MD and forced swimming test

The effects of MD and treatments with *C. asiatica* (30 mg/kg), madecassic acid (10 mg/kg), and escitalopram (10 mg/kg) on the parameters evaluated in the forced swimming test are illustrated in Fig. 4. One-way ANOVA revealed a significant interaction among experimental groups (F = 9,4344; p < 0,0001). Tukey's post hoc test indicated the following differences: MD significantly increased immobility time (p < 0,01), and treatments with *C. asiatica* (p < 0,001), madecassic acid (p < 0,001), and escitalopram (p < 0,01) reversed the effect of MD. Concerning swimming time, one-way ANOVA revealed a significant interaction among groups (F = 3,1520; p < 0,05). However, Tukey's post hoc test revealed that MD tended to reduce swimming time, not reaching statistical significance (p = 0,064). Although all MD-treated groups increased swimming time, post hoc testing revealed a significant increase only for the madecassic acid-treated group compared to the saline-treated MD group (p < 0, 05). There was no statistically significant difference among the groups for climbing in the forced swimming test.

3.1.2 Locomotor Activity

The effects of MD and treatments with *C. asiatica* (30 mg/kg), madecassic acid (10 mg/kg), and escitalopram (10 mg/kg) on the parameters evaluated in the open field test are shown in Fig. 5. In the test, no significant interaction between the stress-free and MD groups. Both MD and treatments did not induce significant changes in locomotor activity, evaluated through the number of crossings and rearing in the test of the open field.

3.3 IL-1β and IL-6 levels in the hippocampus

Is shown in Fig. 6 the levels of IL-1β and IL-6 in the hippocampus. One-way ANOVA revealed a significant interaction among groups, both for IL-1β ($F = 5.98$; $p < 0,01$) and for IL-6 ($F = 6.06$; $p < 0,01$). Treatment with MD + saline increased both IL-β ($p < 0,05$) and IL-6 ($p < 0,01$) compared to the saline control group. Treatments with escitalopram ($p < 0,01$) and madecassic acid ($p < 0,05$) significantly reduced IL-1β levels in the hippocampus in comparison to MD + saline. Likewise, treatment with the classic antidepressant escitalopram ($p < 0,01$), *C. asiatica* ($p < 0,05$), and with madecassic acid ($p < 0,05$) significantly reduced the IL-6 levels in comparison to MD + saline.

3.4 Oxidative stress analysis

3.4.1 MPO activity

The effects of MD and treatments with C. asiatica, madecassic acid, and escitalopram on serum and hippocampal MPO activity are illustrated in Fig. 7. In the serum, one-way ANOVA revealed a significant interaction among groups ($F = 11,28$; $p < 0,0001$). Post hoc analyses revealed that MD + saline increased the MPO activity ($p < 0,0001$) compared to control + saline. Treatment with escitalopram ($p < 0,0001$), *C. asiatica* ($p < 0,0001$), and madecassic acid ($p < 0,0001$) decreased the MPO activity in the serum, in comparison with MD + saline. In the hippocampus, there was no statistical significance among the experimental groups.

3.4.2 TBARS levels

The levels of TBARS in serum and hippocampal after treatments with C. asiatica, madecassic acid, and escitalopram are presented in Fig. 8. In the serum, one-way ANOVA revealed a significant interaction among groups ($F = 8,28$; $p < 0,001$). Post hoc analyses found that MD + saline and *C. asiatica* had significantly increased TBARS levels ($p < 0,0001$) compared to the saline control group. The treatment with escitalopram and madecassic acid decreased TBARS levels in serum ($p < 0,05$). In the hippocampus, one-way ANOVA revealed significant group interaction ($F = 4,77$; $p < 0,01$). MD + saline group presented increased levels of TBARS ($p < 0,05$) compared to the saline group. Groups treated with escitalopram ($p < 0,0001$) and madecassic acid ($p < 0,05$) had decreased TBARS levels compared to MD + saline.

3.4.3 PSH and NPSH levels

In Fig. 9 are presented the results obtained to PSH and NPSH levels on serum and hippocampus. In serum, MD + saline increased the levels of PSH ($F = 5.105$; $p < 0,01$) in comparison to the saline group. When compared to MD + saline, madecassic acid was able to decrease the PSH levels ($p < 0,01$). For NPSH, escitalopram increased levels ($p < 0,01$) in the hippocampus. There was no statistical significance for the others evaluated parameters.

4 Discussion

Several works have shown the potential of *C. asiatica* to be used against neuropathologies, such as in neurodegeneration [58] and neuroimmune diseases [59]. A systematic review with meta-analysis showed that *C. asiatica* can improve alertness and relieve anger, symptoms associated with mood outcomes [60], findings which reinforces the pharmacological application of this plant. Furthermore, a phase 1 clinical study indicated that doses around 250 mg to 500 mg of standardized extract from *C. asiatica* had no collateral effects and was well tolerated in healthy humans [61]. Thus, grounded in these previous robust scientific reports, we evaluated the effect of HECa and madecassic acid in rats submitted to the early-life MD protocol. In an unprecedented manner, we found that HECa and madecassic acid reversed or significantly reduced depressive-like behaviors, inflammation in the hippocampus, and oxidative stress in the serum and hippocampus.

In the first experiment, we induced depressive-like behaviors in rats through MD protocol as described in other studies [27, 62]. The forced swimming test was used to assess depressive-like behaviors. This test is widely used to evaluate the effects of substances with antidepressant potential [63–65]. As predicted, early life MD protocol culminated in depressive-like behaviors in adulthood animals, corroborating the scientific literature reported [62, 66–69].

In parallel, early-life MD groups were treated with HECa (30 mg/kg), madecassic acid (10 mg/kg), and escitalopram (10 mg/kg) to assess its potential for reversion of depressive-like behavior. All treatments were capable of reducing stress-induced depressive-like behaviors. In this context, Sun et al. [70] evidenced that both madecassic acid and asiatic acid (from *C. asiatica*) decreased the immobility time in the forced swim test. Likewise, Kalshetty et al. [40] proved that extract of *C. asiatica* exhibited antidepressant-like effects in a protocol of olfactory bulbectomy in rats. The effect of escitalopram was according to literature since this drug is well-known as a classic antidepressant involved in reducing clinical depression symptoms and depressive-like behaviors in animal models [71–74]. Similar results of improvement in depressive-like behavior were found from the chronic administration of catechin, one of the compounds identified in the analysis of the plant extract, in a model of depression also in rats [75]. It is essential to highlight that this is the first study that observed the antidepressant-like effect of this medicinal species using the MD protocol and the forced swimming test.

The alterations in the parameters of the forced swimming test indicate that the treatment with HECa and madecassic acid contribute positively to reducing depressive-like behaviors caused by MD in rodents, a response possibly mediated by the neuroprotective effect of the administered herbal substances.

MD and pharmacological treatments did not induce changes in open-field mobility parameters. The locomotor activity evaluated in this test is a parameter from the sedative or stimulant effect from stress or treatments [76], so this result indicates that the stress protocol or drugs did not induce a significant sedative or stimulant effect that could interfere with the animals' mobility behaviors.

The reduction in immobility time evaluated in the forced swimming test, induced by HECa and madecassic acid, suggests that this plant and its active compound have a potential antidepressant effect. Research suggests that increased swimming time and decreased immobility time in the forced swimming test are related to the activation of the serotonergic system and the increased time of serotonin in the synaptic cleft. Treatment with classic antidepressants that cause serotonergic modulation reduces immobility time and increases swimming time [77–79].

Although the literature does not have results with protocols similar to this work, a recent study observed that verbascoside (asiaticoside) pointed in this study, and a triterpenoid component of *C. asiatica*, exerted an antidepressant-like effect in mice subjected to chronic moderate stress and reduced the expression of inflammatory cytokines [37].

A recent study provides evidence of the antioxidant, anti-aging, and anti-stress effects of a regular diet containing a composition of *C. asiatica* with vitamins C and D and zinc in an animal model with middle-aged rats [80]. Besides, the medicinal species was considered in this research because it has a neuroprotective potential [58]. In *vitro* research found that the active compound madecassic acid has a strong effect on potentiating telomerase activity [80]. The telomerase enzyme is crucial in preventing telomere shortening and, consequently, inflammation, aging, and cell death, which are also involved in the pathophysiology of MDD [36, 81, 82]. The chronic stress experienced by individuals with MDD culminates in chronic systemic inflammation and, concomitantly, reduces telomerase activity and induces cellular aging [83].

Immune alterations, such as increased levels of IL-1β and IL-6, contribute to the pathophysiology of MDD [84]. Other studies have shown changes in inflammatory mediators in animals that have experienced stressors, such as MD and adulthood chronic stress, and have shown depressive-like behavior in behavioral tests [45, 85]. Hippocampus is a brain region potentially affected by neuroinflammation and is related to memory, learning, and negative feedback regulation to the HPA axis, which interacts and interferes with immune system functions [62, 86, 87]. In this study, we observed that MD significantly increased IL-1β levels in the hippocampus, and the treatments with madecassic acid and escitalopram reversed the effect of MD. Similarly, MD significantly elevated IL-6 levels in the hippocampus, and treatments with HECa, madecassic acid and escitalopram reversed the effect of MD. In this study, the effects of escitalopram are in agreement with the scientific literature, considering that treatment with escitalopram in animal models of depression reverses the depressive-like symptoms induced by the model and reduces the levels of pro-inflammatory cytokines, such as IL-1β, IL-6, TNFα, and INF-γ [33].

The reduction of IL-1β and IL-6 levels in the hippocampus suggests that madecassic acid has anti-inflammatory properties and corroborates the scientific literature. An *in vitro* study found that madecassic acid has anti-inflammatory potential in RAW 264.7 macrophage cells, culminating in the reduction of inducible nitric oxide synthase (iNOS), COX-2, TNF-α, IL-1β, and IL-6 mRNA expression [88]. In addition, research carried out in diabetic mice showed that the administration of madecassic acid chronically caused a reduction in the levels of IL-1β and IL-6 in the kidneys and hearts of the animals

[89]. In diabetic rats, chronic treatment with *C. asiatica* decreased renal levels of MDA, TNF-α, and interferon-γ (IFN-γ) in the kidneys and brain, reinforcing the inflammatory effect of the species [90].

Verbascoside, one of the compounds found by staying present in the HECa from this study, has been described as a potent reducer of pro-inflammatory cytokine in neuropathologies, mainly by the suppression of IL-1β and IL-6 [91]. Another compound from HECa in this research that may explain the anti-inflammatory effect is chicoric acid. It has been related to preventing neurodegeneration in the striatum of mice by regulation of IL-17, IFN-γ, and transforming growth factor beta (TGF-β), as well as mitigating dopaminergic neuronal lesions [91]. All these results corroborate the anti-inflammatory effect of HECa against neurodegeneration, as occurs in depressive-like disease.

Anti-inflammatory therapies are being widely researched for treating MDD and other psychiatric pathologies [92], and non-pharmacological treatments are therapeutic strategies to control depression. An example is regular physical exercise, which causes a response comparable to conventional therapies, individually or as an adjuvant to pharmacological therapy. Physical activities control depressive symptoms by increasing anti-inflammatory factors and processes and decreasing circulating proinflammatory substances, controlling the neuroinflammation present in the pathophysiology of MDD [87]. These results highlight the importance of expanding investigations into the potential antidepressant effects, at least partly from the anti-inflammatory properties of *Centella asiatica.*

The pathophysiology of MDD is closely related to oxidative stress. Furthermore, oxidative stress is closely related to neuroinflammation [93]. High levels of protein carbonylation and nitric oxide, as well as reduced SOD and glutathione, were observed in elderly individuals with MDD [94]. Interestly, chronic treatment with *C. asiatica* induced free radical reduction/triggered lipid peroxidation, maintained an adequate level of antioxidant enzymes in hippocampus, in animals chronically exposed to aluminum chloride ($AlCl_3$) [95]. Given these statements, we finally evaluated the redox profile of rats after and before of treatments with HECa, madecassic acid and escitalopram. Animals that underwent MD had an increase in seric MPO levels, and increase in seric and hippocampal TBARS levels, showing that MD favors pro-oxidant conditions. Treatments with HECa, madecassic acid and escitalopram reversed these alterations. In the hippocampus, TBARS in serum and hippocampus, treatments with escitalopram and madecassic acid reversed the change (Fig. 8).

Studies indicate that the antioxidant effects of plants may be related to their anti-inflammatory effect, and in this research, *C. asiatica* demonstrated an anti-inflammatory effect on IL-1 and IL-6. The scientific literature points to evidence that oxidative stress is positively associated with neuroinflammation and *C. asiatica* is therapeutic potential in these situations [90, 96]. *In vitro* and *in vivo* analyses indicate that the plant's triterpenes contribute to the antioxidant, cholinesterase inhibitory activity, and antiamnesic effect of *C. asiatica*. Still, they are not the only substances with this effect in the extract [96]. Considering research on bioavailability, distribution, and antioxidative effects, it is possible to hypothesize that the *C. asiatica* extract did not reverse the increase in TBARS because it did not have sufficient amounts of the active compound madecassic acid, which had beneficial effects on MDA levels [97]. Still, catechin, a

substance in the extract of *C. asiatica* used in this study, has an antioxidant potential identified in a study with obese adults. This substance was related to reducing glutathione peroxidase (GPX) levels, an essential reduction in oxidative stress in the body [98]. Another substance in the extract is ellagic acid, which reduces oxidative stress in women with polycystic ovaries [99]. In this study, there was less catechin and ellagic acid than needed for the antioxidative effect. Therefore, these results indicate that the active compound madecassic acid has potential antioxidant action to abrogate the oxidative stress.

As we found that MD protocol also induced, associated with depressive-like behaviors and inflammation, a pro-oxidant state, we searched for signals of antioxidant biomarkers. In this sense, some important antioxidant molecules involved in redox balance are those belongs to the thiols system, which is characterized by the organic sulfur derivatives known as sulfhydryl groups (-SH), such as glutathione (GSH) [100]. In this study, MD impacted in seric PSH levels with significant increase. On the other hand, the treatment with madecassic acid was capable of decreasing levels of PSH while other treatments had no effects in this biomarker (Fig. 9). It is well-known that sulfhydryl groups act as scavengers of molecules [101]. Thus, a possible explanation for increase in PSH levels is that under stress-induced by MD, this endogenous antioxidants defense increase in a homeostatic attempt to abrogate the levels of pro-oxidants molecules. In the case of treatments, the madecassic acid itself played the antioxidant role, with a reduction in PSH close to the control baselines levels in this group.

In addition, we also found increased levels of NPSH in treatment with escitalopram in the hippocampus (Fig. 9). This result is supported by several works, as shown that escitalopram suppressed the effects of increased oxidative stress, with decreasing in MDA levels in the hippocampus and increasing GSH both in the hippocampus and prefrontal cortex, as well ass alleviated stress-induced depressive and anxious behaviors in rats [102]. A study performed by Cimen et al. [103], in which subchronic treatment of patients with escitalopram modulates both oxidants and antioxidants elements leading to close health individuals.

The behavioral results, inflammatory and redox biomarkers that we observed in the this study, as well as the results in the scientific literature on the biological actions of the species *C. asiatica* and its active compound, madecassic acid, highlight the importance of continuity in the analysis of the anti-inflammatory, antioxidant and antidepressant profile.

5 Conclusion

MD stress in the first days of life induced a significant increase in depressive-like behaviors in adulthood. The animals submitted to MD stress showed a significant increase in inflammatory cytokines, IL-1β and IL-6, in the hippocampus, and a significant increase in the MPO, in the serum, and TBARS, in the serum and hippocampus, suggesting that the stress in childhood induces neuroinflammation and oxidative stress throughout life. Treatments with *C. asiatica* extract, active compound madecassic acid, and the antidepressant escitalopram reversed or reduced depressive-like behaviors and levels of inflammatory cytokines in the hippocampus. These results strongly suggest that the medicinal species *C. asiatica* and

its active compound have antidepressant potential and that the reduction of hippocampal neuroinflammation and oxidative stress in serum and hippocampus are mechanisms involved in the antidepressant-like effect of the species. There are still no studies in the literature that evaluate the effect of *C. asiatica* and madecassic acid in humans on inflammatory markers. Must be carried out to identify and elucidate the mechanisms by which *C. asiatica* and the active compound madecassic acid have antidepressant, anti-inflammatory, and antioxidant potential in the animal model of MD.

Declarations

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Authors' contributions: All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Amanda Gollo Bertollo, Maiqueli Eduarda Dama Mingoti, Jesiel de Medeiros, Gilnei Bruno da Silva, Giovana Tamara Capoani, Heloisa Lindemann, Joana Cassol, Daiane Manica, Tacio de Oliveira, Michelle Lima Garcez, Margarete Dulce Bagatini, Lilian Caroline Bohnen, Walter Antônio Roman Junior, and Zuleide Maria Ignácio. The first draft of the manuscript was written by Amanda Gollo Bertollo and all authors commented on previous versions of the manuscript. Zuleide Maria Ignácio reviewed and supervised. All authors read and approved the final manuscript.

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Figures

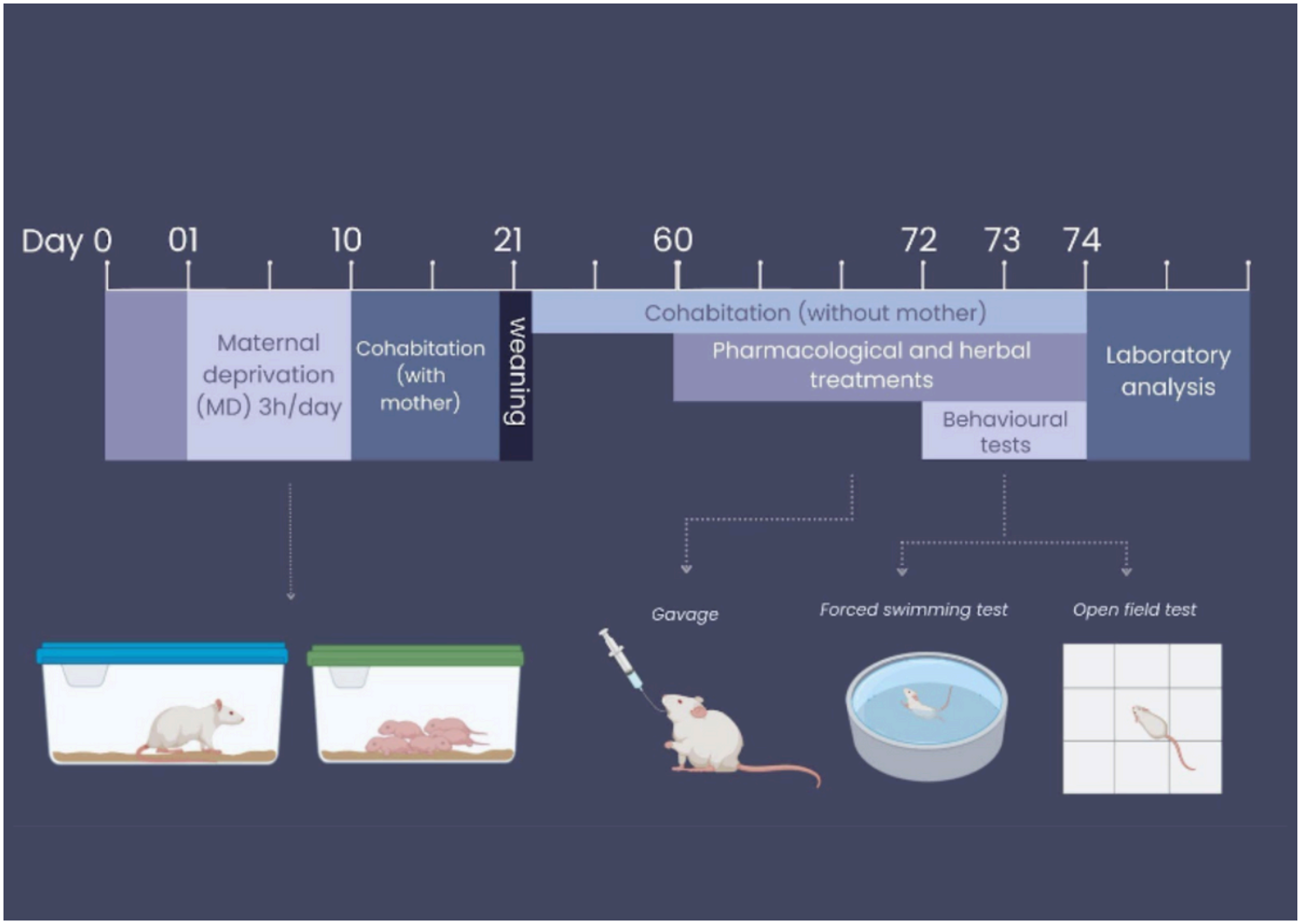

Figure 1

Experimental design of the MD protocol, pharmacological treatments, and behavioral tests.

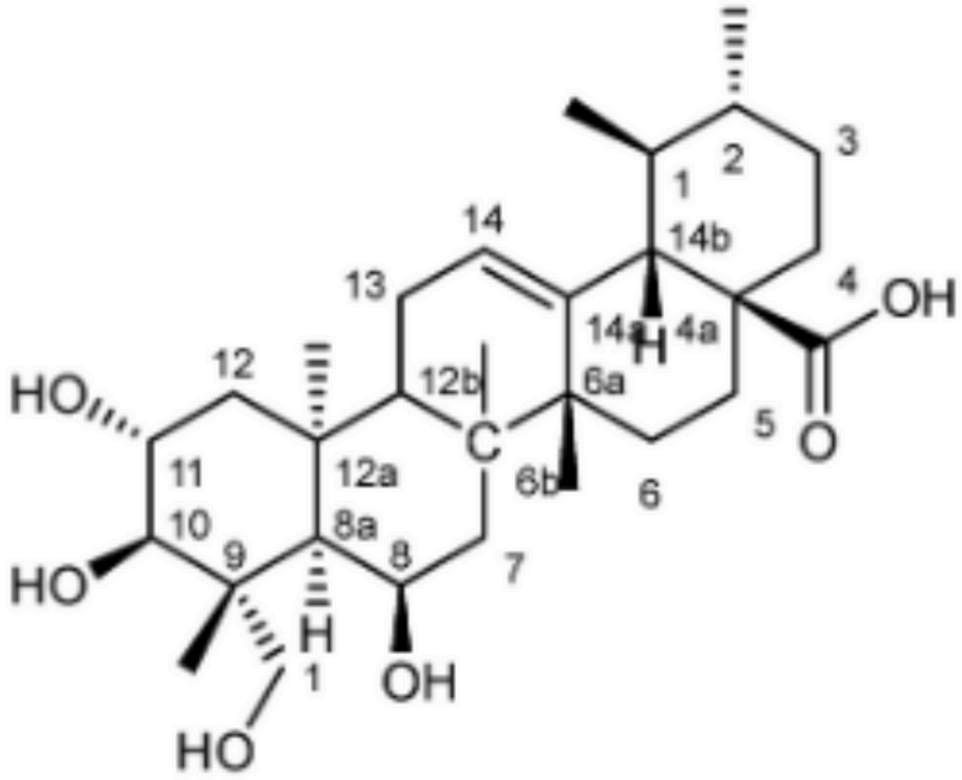

Figure 2

Chemical structures of Madecassic acid.

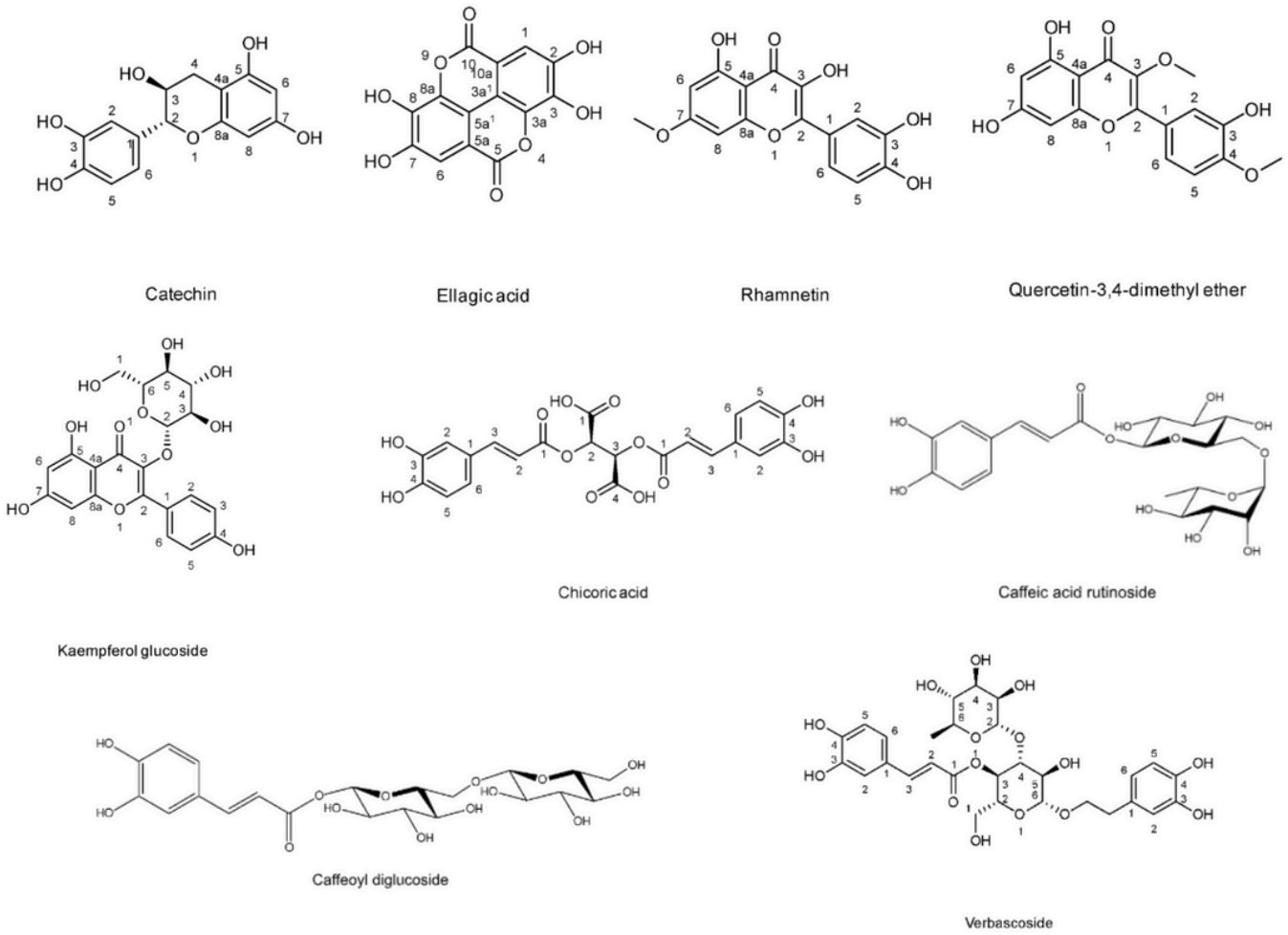

Figure 3

Chemical structures denoted for the hydroalcoholic extract from Centella asiatica (HECa).

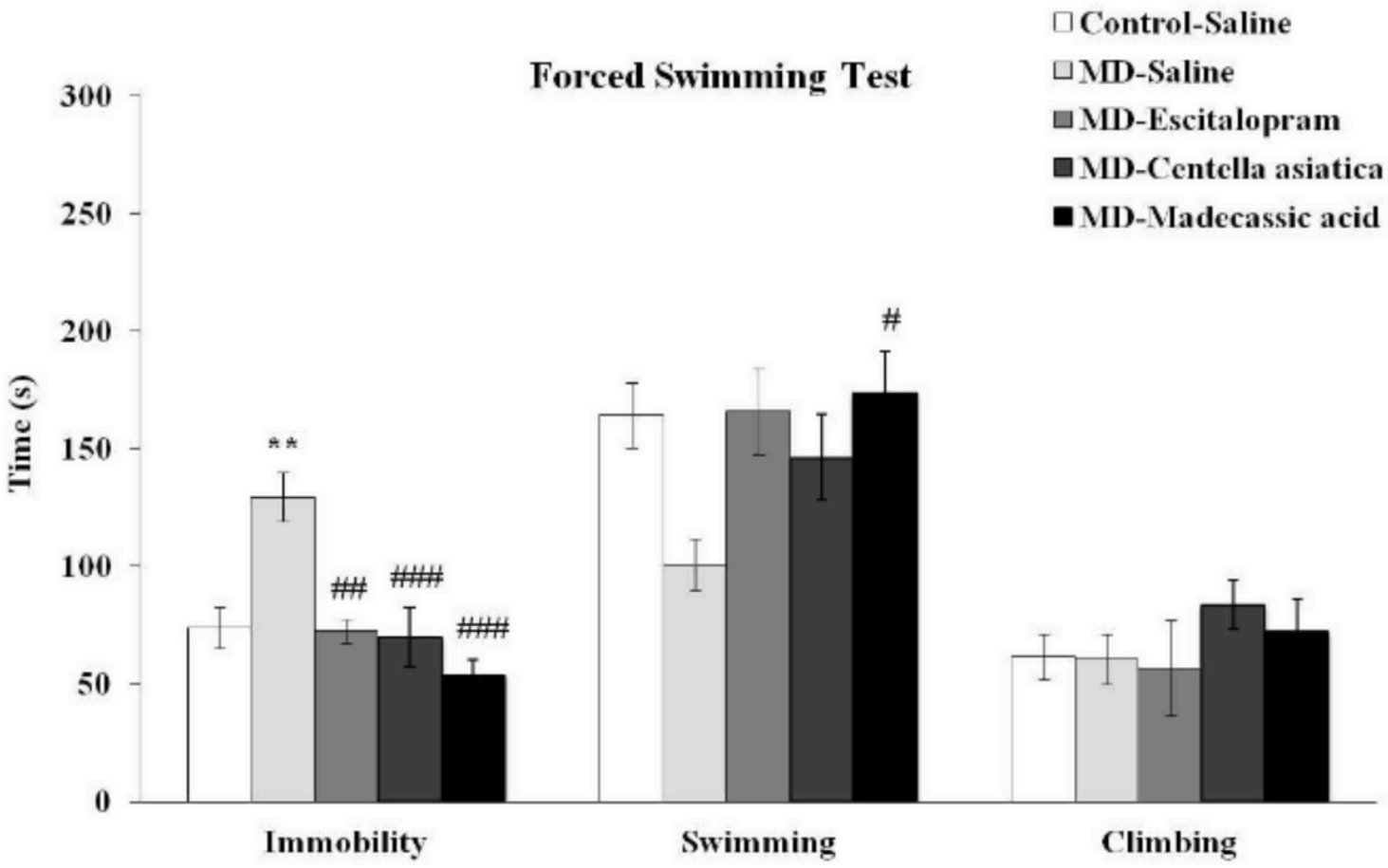

Figure 4

Effects of MD stress and treatments with hydroalcoholic extract from *Centella asiatica* (HECa, 30 mg/kg), madecassic acid (10 mg/kg), and escitalopram (10 mg/kg) on mobility parameters inthe forced swim test. Data are presented as the mean ± standard error of the mean. **statistical difference between Control Saline and MD Saline ($p < 0,01$); # different from MD Salina ($p < 0,05$); ##different from MD Salina ($p < 0,01$); ### different from MD Salina ($p < 0,001$).

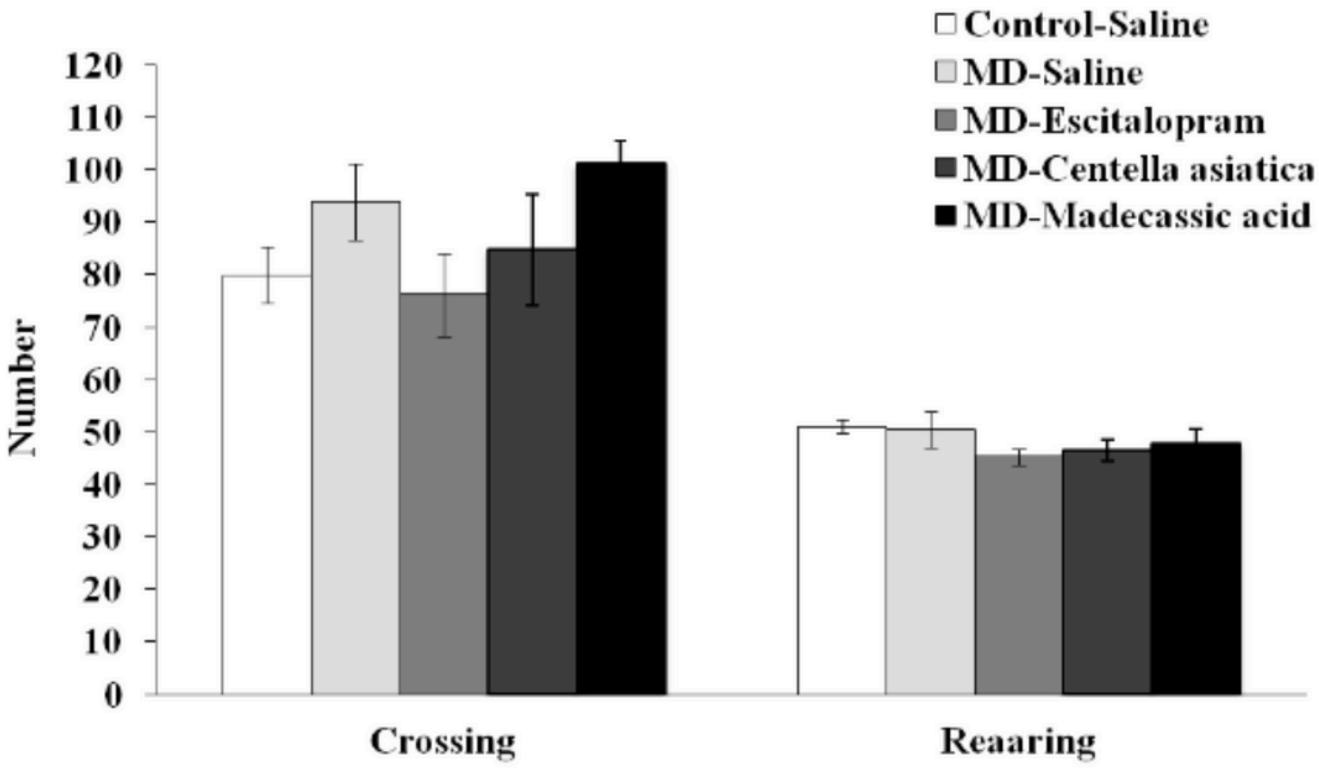

Figure 5

Effects of MD and treatments with hydroalcoholic extract from *Centella asiatica* (HECa, 30 mg/kg) madecassic acid (10 mg/kg), and escitalopram (10 mg/kg) on exploratory motor activity. Data are presented as the mean ± standard error of the mean.

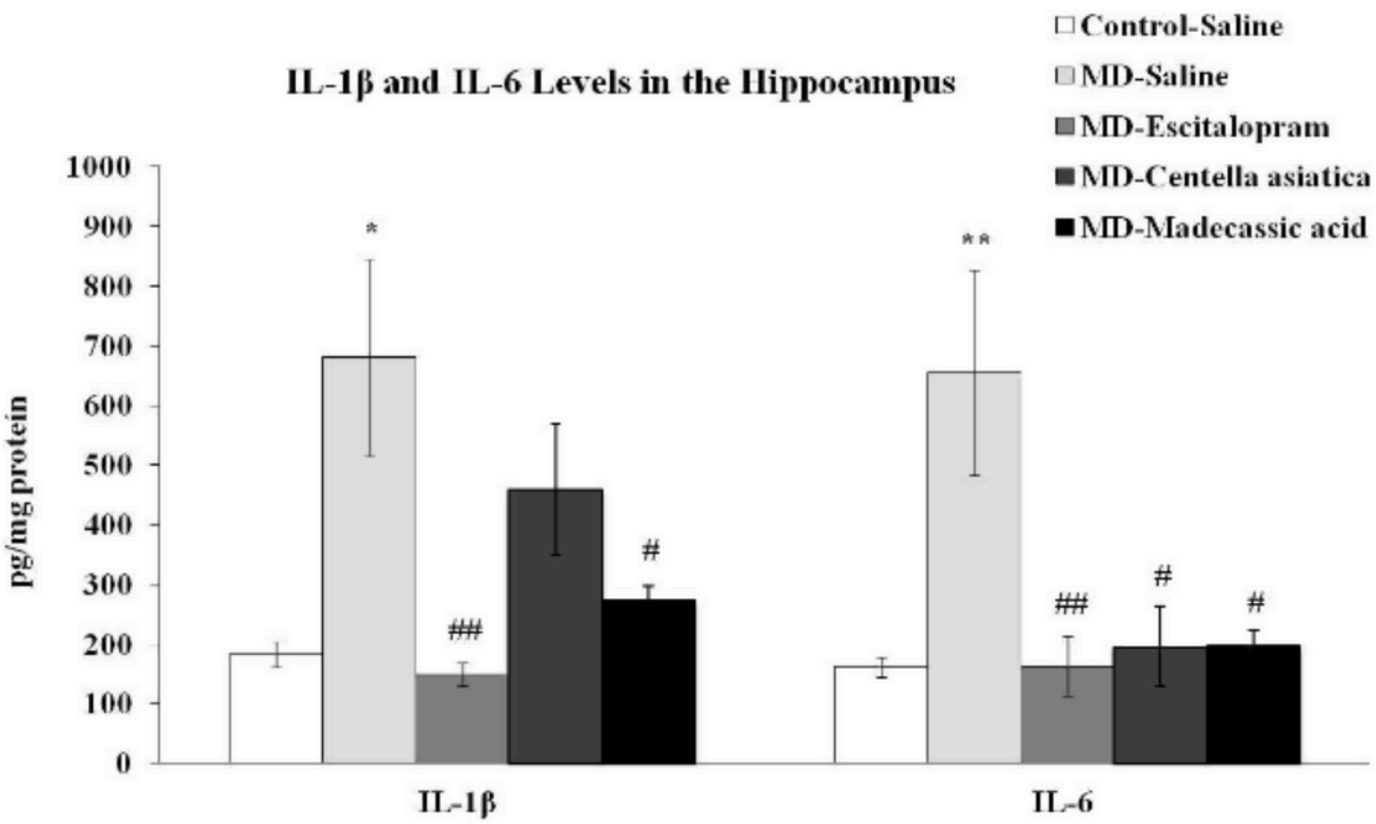

Figure 6

Effect of MD and treatments with hydroalcoholic extract from Centella *asiatica* (HECa, 30 mg/kg), madecassic acid (10 mg/kg), and escitalopram (10 mg/kg) on IL-1β and IL-6 levels in the hippocampus. Data are presented as the mean ± standard error of the mean. *statistical difference between Control Saline and MD Saline ($p < 0,05$); **statistical difference between Control Saline and MD Saline ($p < 0,01$); #different from saline MD ($p < 0,05$); ## different from saline MD ($p < 0,01$); ### different from saline MD ($p < 0,001$).

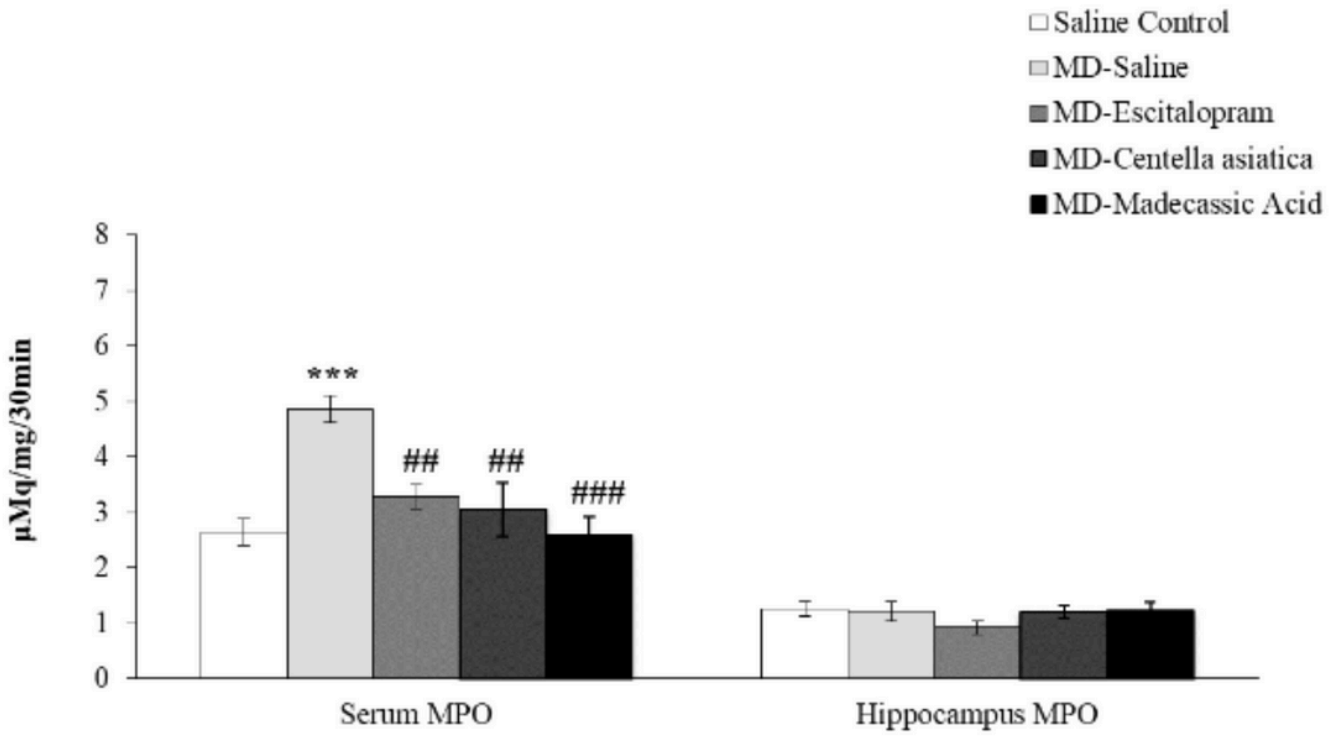

Figure 7

Effects of MD stress and treatments with hydroalcoholic extract from Centella *asiatica* (HECa, 30 mg/kg), madecassic acid (10 mg/kg), and escitalopram (10 mg/kg) on serum and hippocampal myeloperoxidase (MPO) activity. Data are presented as the mean ± standard error of the mean. ***different from the Saline Control ($p < 0,0001$); ##different from MD Salina ($p < 0,01$); ### different from MD Salina ($p < 0,0001$).

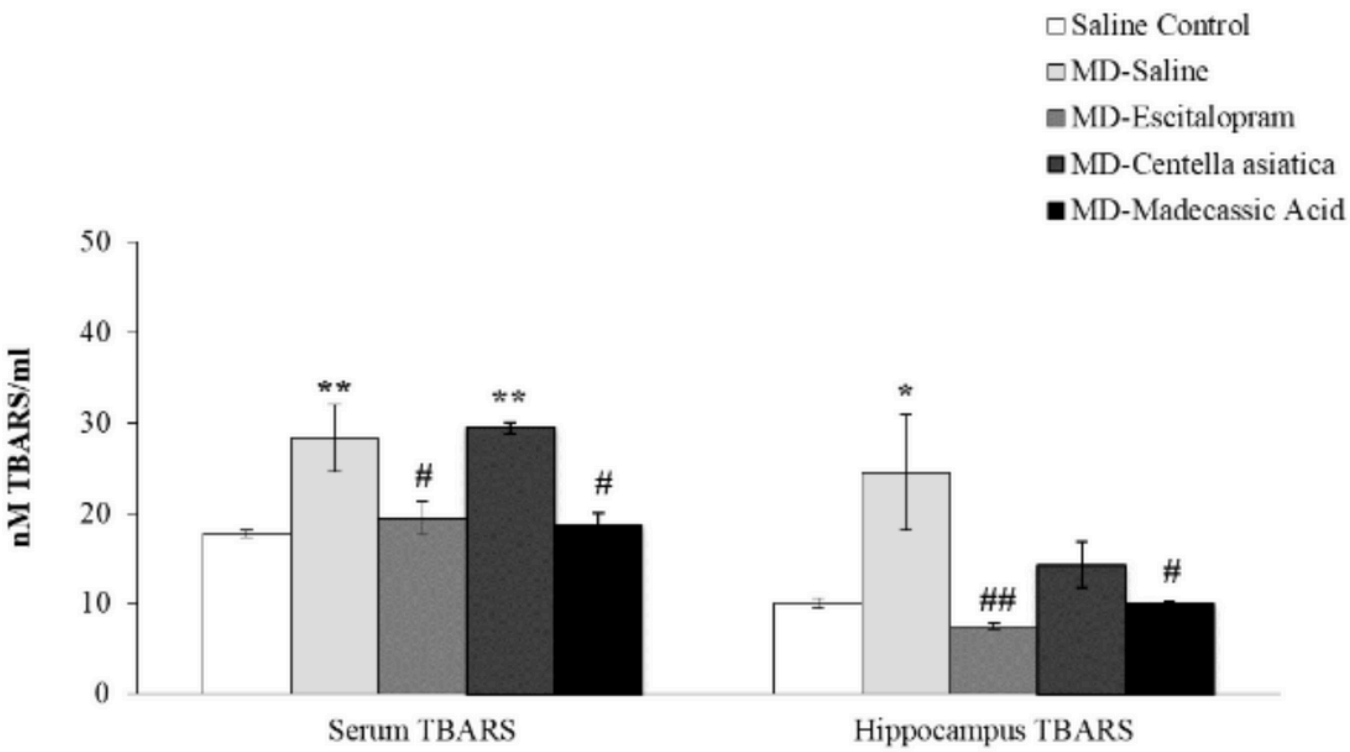

Figure 8

Effects of MD stress and treatments with hydroalcoholic extract from Centella *asiatica* (HECa, 30 mg/kg), madecassic acid (10 mg/kg), and escitalopram (10 mg/kg) on the levels of thiobarbituric acid reactive substances (TBARS) in the serum and hippocampus. Data are presented as the mean ± standard error of the mean. *different from the Saline Control ($p < 0,05$); **different from the Saline Control ($p < 0,01$); #different from MD Salina ($p < 0,05$); ##different from MD Salina ($p < 0,01$).

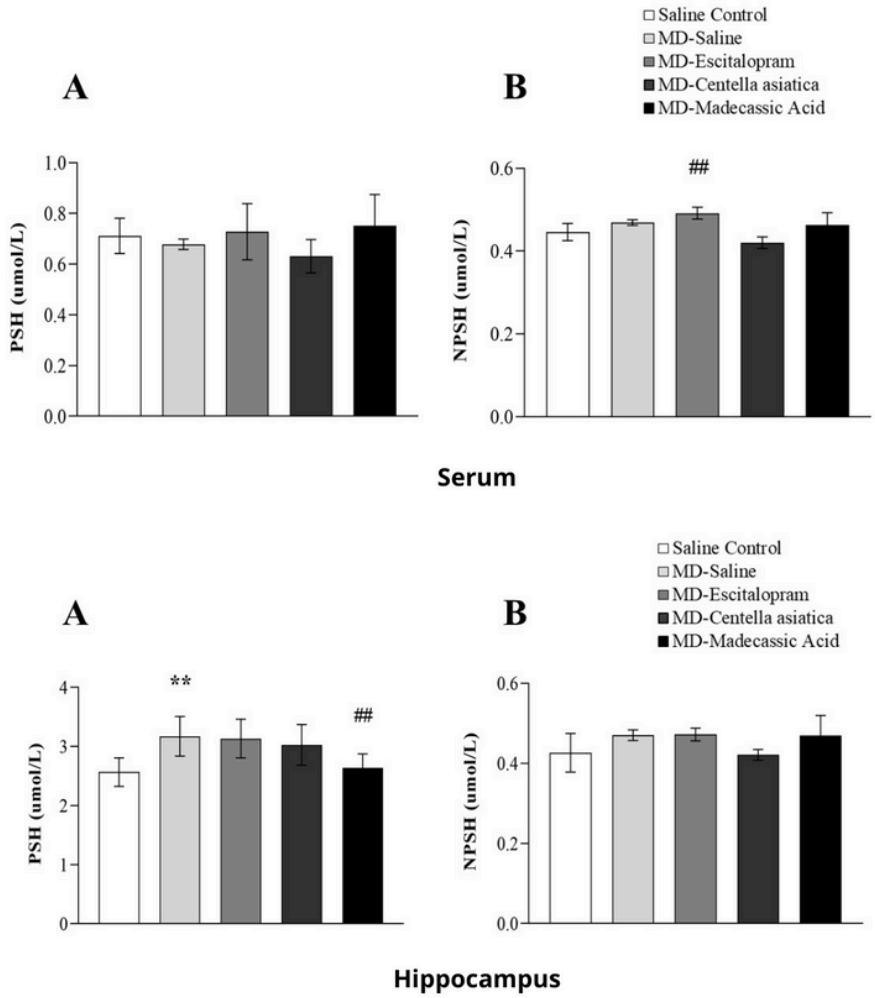

Figure 9

Effects of MD stress and treatments with hydroalcoholic extract from *Centella asiatica* (HECa, 30 mg/kg), madecassic acid (10 mg/kg), and escitalopram (10 mg/kg) on the levels of total thiols (PSH) and non-protein thiols (NPSH) in the serum (A) and hippocampus (B). Data are presented as the mean ± standard error of the mean. *different from the Saline Control ($p < 0,05$); **different from the Saline Control ($p < 0,01$); #different from MD Salina ($p < 0,05$); ##different from MD Salina ($p < 0,01$).